

RESEARCH PAPER

# Characterization of a transport activity for long-chain peptides in barley mesophyll vacuoles

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## Abstract

The plant vacuole is the largest compartment in a fully expanded plant cell. While only very limited metabolic activity can be observed within the vacuole, the majority of the hydrolytic activities, including proteolytic activities reside in this organelle. Since it is assumed that protein degradation by the proteasome results in the production of peptides with a size of 3–30 amino acids, we were interested to show whether the tonoplast exhibits a transport activity, which could deliver these peptides into the vacuole for final degradation. It is shown here that isolated barley mesophyll vacuoles take up peptides of 9–27 amino acids in a strictly ATP-dependent manner. Uptake is inhibited by vanadate, but not by  $\text{NH}_4^+$ , while GTP could partially substitute for ATP. The apparent affinity for the 9 amino acid peptide was 15  $\mu\text{M}$ , suggesting that peptides are efficiently transferred to the vacuole *in vivo*. Inhibition experiments showed that peptides with a chain length below 10 amino acids did not compete as efficiently as longer peptides for the uptake of the 9 amino acid peptide. Our results suggest that vacuoles contain at least one peptide transporter that belongs to the ABC-type transporters, which efficiently exports long-chain peptides from the cytosol into the vacuole for final degradation.

**Key words:** ABC transporter, peptide transport, transport, vacuole.

## Introduction

Plant vacuoles are multifunctional organelles that play a central role during the whole of a plant's life. While young cells often contain many small vacuoles, most of the mature cells have one central vacuole, which occupies up to 90% of the cell volume. In addition, small vacuoles may be present in these cells. A large number of substances such as inorganic ions, soluble carbohydrates, organic acids, amino acids, secondary compounds or modified xenobiotics, but also hydrolytic and biosynthetic enzymes, can be found within vacuolar compartments. The vacuole is responsible for numerous processes, sometimes unique to the plant cell (Hörtensteiner and Feller, 2002; Martinoia *et al.*, 2007). The more negative water potential drives the uptake of water into the large central vacuole, generating the turgor pressure

that enables the cells to expand, as well as to sustain the shape of non-lignified plants. The vacuole can serve as a transient storage compartment for nutrients, which can be released when required for growth and development. Furthermore, in specialized storage tissues or seeds, the vacuole can act as a storage compartment for protein. The vacuole also plays a crucial role in the tight control of cytosolic concentrations of metabolites and ions for cell homeostasis. In addition, the vacuole is implicated in detoxification processes and defence responses by accumulating and sequestering toxic compounds and defence molecules.

To facilitate the intensive exchanges that occur between the cytosol and the vacuole a large number of proteins are

embedded in the vacuolar membrane. Two proton pumps, namely the H<sup>+</sup>-ATPase (V-ATPase) and the H<sup>+</sup>-pyrophosphatase (V-PPase), located in all the vacuolar membranes, generate the electrochemical gradient required to energize the large majority of the transport mediated by tonoplast proteins (Sze *et al.*, 1999; Maeshima, 2001). Two other classes of transporters mediate a directly ATP energized uptake and allow the accumulation of their substrates in the vacuole independently of the proton motive force: cation pumps belonging to the class of the P-type ATPases and ATP-binding cassette (ABC) transporters (White and Broadley, 2003; Rea, 2007; Yazaki *et al.*, 2009).

The ATP-binding cassette (ABC) transporters constitute a large, diverse, ubiquitous superfamily, widespread in bacteria, fungi, animals, and plants. Most of the ABC proteins are involved in the directly energized transport of a large variety of substances across various biological membranes. Compared with all other organisms, plants contain by far the most ABC transporters. Most of them can be grouped into the eight major subfamilies (A–H), universally shared by living organisms (Verrier *et al.*, 2008). In plants, ABC proteins have been shown to be implicated in diverse essential processes such as the vacuolar sequestration of xenobiotics, heavy metal tolerance, pigment accumulation, auxin transport, alkaloid import at the plasma membrane, wax deposition on the cuticle, lipid catabolism, stomatal regulation, disease/pathogen resistance or assembly of redox-active cytosolic Fe/S proteins (Martinoia *et al.*, 1993; Lu *et al.*, 1998; Gaedeke *et al.*, 2001; Shitan *et al.*, 2003; Pighin *et al.*, 2004; Xu and Møller, 2004; Lee *et al.*, 2005; Geisler and Murphy, 2006; Kim *et al.*, 2007; Rea, 2007; Yazaki *et al.*, 2009). However, the number of uncharacterized ABC transporters remains high and their implication in plant metabolism still awaits discovery.

Peptides are implicated in many different processes occurring in living cells. In mammals they are used, for example, for antigen processing or as hormones (Parcej and Tampe, 2007). Furthermore, some antibiotics and anticancer drugs are peptides (Dantzig *et al.*, 1992; Hori *et al.*, 1993). In bacteria, peptides can be involved in quorum sensing (Swift *et al.*, 1996). In plants, during seed germination, small peptides are produced to supply the seedling with reduced nitrogen (Higgins and Payne, 1981; Stacey *et al.*, 2002b). Peptide transporters were identified in three gene families. Members of the peptide transporter/nitrate transporter 1 (PTR/NRT1) family recognize di- and tripeptides, while some members of the oligopeptide transport (OPT) family transport predominantly tetra- and pentapeptides, though longer or shorter peptides are sometimes also translocated (Tsay *et al.*, 1993; Rentsch *et al.*, 1995, 2007; Koh *et al.*, 2002; Stacey *et al.*, 2002a; Waterworth and Bray, 2006; Pike *et al.*, 2009). In mammals, three members of the ABC transporter superfamily, the so-called TAPs, have been shown to be involved in the transport of longer chain peptides. They reside either in the ER, Golgi or in lysosomal membrane and mediate the transport of peptides ranging from 6–59 amino acids (Kelly *et al.*, 1992; Lankat-Buttgereit and Tampé, 2003; Wolters *et al.*, 2005).

Early work on plant vacuoles has shown that vacuoles contain highly active proteases (Boller and Kende, 1979). It was suggested that these proteases are implicated in cellular protein turnover. However, this hypothesis still awaits confirmation. Proteomic studies using Arabidopsis and barley vacuolar membranes have revealed a large number of putative vacuolar transporters with still unknown function, including also TAP homologues (Carter *et al.*, 2004; Endler *et al.*, 2006; Jaquinod *et al.*, 2007). It was therefore of interest to investigate whether vacuoles exhibited peptide transport activity for larger peptides of a size between 9 and 30 amino acids.

## Materials and methods

### Plant material

Barley (*Hordeum vulgare* L. cv. Baraka) was grown in a controlled environment chamber [16/8 h light (300 μE m<sup>-2</sup> s<sup>-1</sup>)/dark cycle at 22 °C and 60% relative humidity].

### Isolation of intact vacuoles

Barley mesophyll vacuoles were isolated using a slight modification of the method described in Rentsch and Martinoia (1991). After resuspension of the protoplasts, they were collected by centrifugation (10 min, 200 g) on a cushion of osmotically stabilized Percoll [500 mM sorbitol, 1 mM CaCl<sub>2</sub> (Fluka Buchs, Switzerland), and 20 mM MES, pH 6]. Protoplasts were suspended in 5 ml of a solution containing 500 mM sorbitol, 30% (v/v) Percoll, 1 mM CaCl<sub>2</sub>, and 20 mM MES, pH 6. This suspension was overlaid with 10 ml medium A [430 mM sorbitol, 25% (v/v) Percoll, 30 mM K-gluconate (Fluka Buchs, Switzerland), and 20 mM HEPES–KOH pH 7.2] and 5 ml medium B (430 mM sorbitol, 30 mM K-gluconate, and 20 mM HEPES–KOH pH 7.2). After centrifugation for 10 min at 200 g, protoplasts were recovered from the upper interface, mixed with 15% (v/v) medium B including 10 mM EDTA and forced through a syringe without a needle to liberate the vacuoles. The protoplasts lysate was suspended in a solution containing 500 mM sorbitol, 20% (v/v) Percoll, and 20 mM HEPES–KOH pH 7.2. The suspension was overlaid with 5 ml of medium B containing 0.1% (w/v) BSA (Fluka Buchs, Switzerland) and 0.2 mM DTT, and 1 ml medium C (400 mM glycine-betaine, 30 mM K-gluconate, and 20 mM HEPES–KOH pH 7.2), including 0.1% (w/v) BSA and 0.2 mM DTT. After centrifugation for 3 min at 200 g and 5 min 1000 g, vacuoles were recovered from the upper interface. The last purification gradient was repeated with the recovered vacuoles, but centrifugation was 5 min at 1000 g. The purified vacuoles recovered from the upper interface were completed with Percoll-solution [to a final concentration of 10% (v/v) Percoll, 500 mM sorbitol, and 20 mM HEPES–KOH pH 7.2] and the suspension was directly used for uptake experiments.

### Uptake experiments

Uptake of fluorescein(φ)-labelled peptides of 9 aa, RRYC(φ)KSTEL; 18 aa, RRYQKSTELRRYC(φ)KSTEL; and 27 aa, RRYQKSTELRRYQKSTELRRYC(φ)KSTEL (Uebel *et al.*, 1997) was measured by a slight modification of the method described by Martinoia *et al.* (1993). For each condition and time point, five polyethylene microcentrifugation tubes (400 μl capacity) were prepared as follows: 70 μl of basal medium (22% Percoll pH 7.2, 430 mM sorbitol, 30 mM K-gluconate, 20 mM HEPES–KOH pH 7.2, 0.1% BSA, and 0.2 mM DTT) containing 0.1 μCi <sup>3</sup>H<sub>2</sub>O, 2 μM of one of the fluorescein-labelled peptides and solutes as indicated in the figures were placed in the bottom of the tubes. Experiments were started by the addition of 30 μl of the vacuole suspension. Samples were

rapidly overlaid with 200  $\mu$ l silicone oil AR 200 and 60  $\mu$ l medium C described above. Incubation was interrupted by centrifugation at 10 000  $g$  for 20 s. The fluorescent peptides contained in medium C were quantified using a fluorescence plate reader ( $\lambda$  ex/em 485/520 nm; Fusion, Packard).  $^3\text{H}_2\text{O}$  equilibrates rapidly between the medium and the vacuolar space and was used to quantify the number of vacuoles by scintillation counting.

For competition experiments, randomized peptide libraries were added (Uebel *et al.*, 1997).

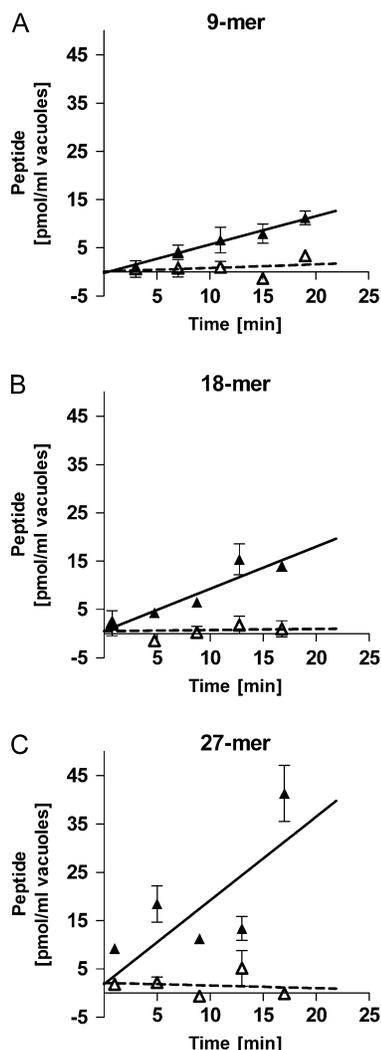
## HPLC analysis

Iodoacetamidefluorescein, the fluorescent-labelled 27 aa peptide and the corresponding degradation products were analysed by C18 reversed phase HPLC (PerfectSil-300-ODS-C18, 250 $\times$ 4.6 mm, 5  $\mu$ m particle diameter, MZ Analysentechnik, Mainz, Germany) using 100 mM ammonium acetate, pH 6.5 as solvent A and 50% (v/v) acetonitrile in 100 mM ammonium acetate, pH 6.5 as solvent B (linear gradient from 15–55% B in 20 min, flow rate of 1 ml  $\text{min}^{-1}$ ). Fluorescent products were detected by a fluorescence detector (Shimadzu RF 935) with ex/em=470/520 nm.

## Results

### Time-dependent uptake of fluorescein-labelled peptides

To characterize a hypothetical vacuolar peptide transporter in plants, highly purified barley mesophyll vacuoles were isolated. Transport assays were performed using three peptides of different size, i.e. a 9 amino acid (aa) (RRYC( $\phi$ )KSTEL), an 18 aa (RRYQKSTELRRYC( $\phi$ )KSTEL), and a 27 aa (RRYQKSTELRRYQKSTELRRYC( $\phi$ )KSTEL) peptide, where  $\phi$  symbolizes the fluorescein fluorophore. Uptake experiments were performed using a peptide concentration of 2  $\mu$ M in the presence or absence of 4 mM MgATP. Uptake of all three peptides was strictly MgATP-dependent (Fig. 1A, B, C). In the absence of MgATP, no increase in fluorescence could be observed, while in the presence of MgATP, efficient peptide uptake occurred. This peptide uptake was linear for at least 20 min. The ATP-dependent transport rates increased with the size of the peptide. They were  $0.59 \pm 0.14$ ,  $0.88 \pm 0.13$ , and  $1.78 \pm 0.50$  pmol  $\text{ml}^{-1} \text{min}^{-1}$  for the 9, 18, and 27 amino acid peptides, respectively. To prove that the peptides and not the free fluorescein is accumulated in the vacuoles, the vacuolar samples were analysed using HPLC (Fig. 2). Iodoacetamidefluorescein, the compound used to label the peptides is eluted after 18 min (Fig. 2A), Iodoacetamidefluorescein is hydrolysed to acetamidefluorescein in water. Due to unknown reasons, two hydrolysis products are formed (Fig. 2A). For the 27 aa peptide, several products could be detected, probably due to different intramolecular interactions. Three predominant peaks were observed at 8.4, 9.6, and 13.7 min (Fig. 2B). Products with the same retention time could be detected in vacuoles, which were incubated for 18 min in the presence of the 27 aa peptide, and separated from the incubation solution prior to analysis (Fig. 2C). One additional peak, probably corresponding to a hydrolysis product of iodoacetamidefluorescein, emerged (Fig. 2C). As shown in Fig. 2D, the HPLC profile changed when vacuoles were kept at room temperature for 45 min after incubation with the



**Fig. 1.** Time-dependent uptake of peptides into isolated barley mesophyll vacuoles. Vacuoles were incubated with 2  $\mu$ M of three different fluorescein( $\phi$ )-labelled peptides; 9 aa: RRYC( $\phi$ )KSTEL (A), 18 aa: RRYQKSTELRRYC( $\phi$ )KSTEL (B), and 27 aa: RRYQKSTELRRYQKSTELRRYC( $\phi$ )KSTEL (C) in the absence (white triangles) or presence (black triangles) of 4 mM MgATP. Peptide transport was quantified by fluorescence detection as described in the Materials and methods. At the time points indicated, incubation was interrupted by removing vacuoles from the incubation medium via centrifugation through silicon oil. Each time point is the mean of five replicates, and error bars represent SD.

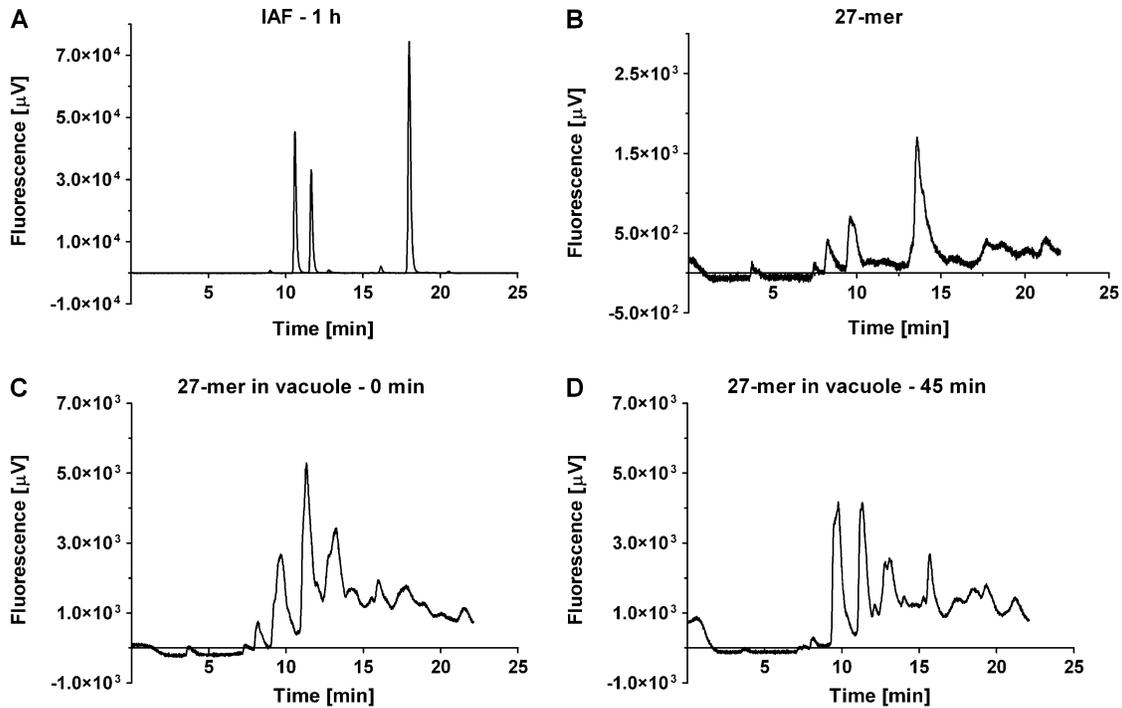
fluorescent peptide. These results indicate that peptides are taken up by vacuoles and readily degraded by proteases present in the vacuole. However, these experiments cannot entirely rule out the possibility that a low amount of free fluorescein is taken up by vacuoles.

### Energization of the peptide transport

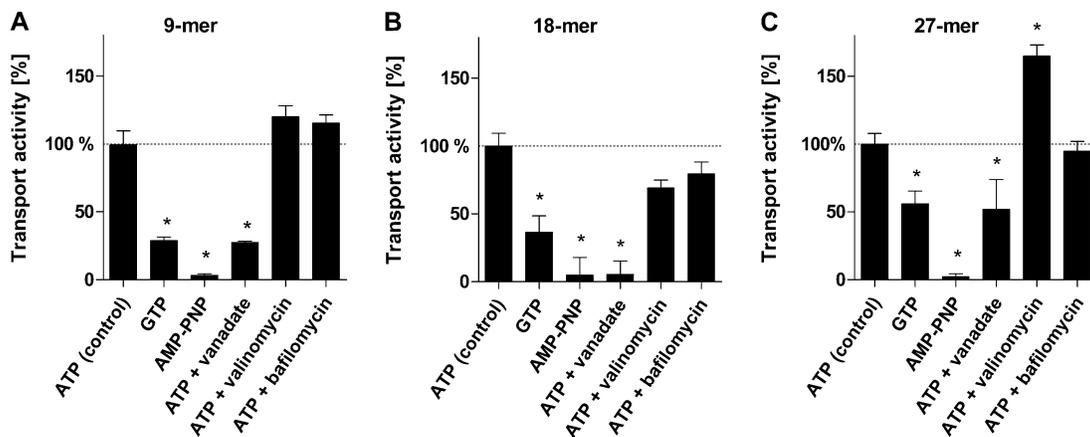
Vacuoles have an acidic pH, therefore, the ATP-dependent peptide transport into barley vacuoles might occur by a secondary energized mechanism utilizing the proton motive force. Alternatively, direct MgATP-hydrolysis by

ABC-type transporters may energize uptake. To distinguish between these two mechanisms, transport experiments were performed using different inhibitors. Since uptake was linear for at least 20 min, peptide uptake rates were determined by using the values for 2 min and 20 min. This allowed correcting for unspecific adsorption. Compared with transport rates in the presence of MgATP (100%), MgGTP, which is known to be able partially to substitute

for MgATP in the case of ABC transporters, was able to catalyse peptide transport to 30–50% (Fig. 3). Interestingly, MgGTP was more efficient in energizing transport of the longer peptides. The non-hydrolysable ATP analogue, AMP-PNP failed to catalyse uptake, indicating that energy is required to drive the transport of these peptides. Valinomycin, a  $K^+$ -specific ionophore, which dissipates the membrane potential, and bafilomycin, a highly specific



**Fig. 2.** HPLC analysis of peptides in vacuolar fractions. HPLC profiles of: iodoacetamidofluorescein after 1 h incubation in water (A), the 27 aa peptide (B), vacuoles incubated for 18 min with the 27 aa peptide and directly subjected to HPLC after separation from the incubation medium (C), and vacuoles which after 18 min incubation with the 27 aa peptide and purification were kept for 45 min in medium B at room temperature (D).



**Fig. 3.** Effect of nucleotides and inhibitors on peptide uptake. Comparison of the transport activities of labelled 9 aa (A), 18 aa (B), and 27 aa (C) peptides in response to nucleotides (MgATP, MgGTP, and AMP-PNP, 4 mM) and inhibitors (0.5 mM vanadate, 10  $\mu$ M valinomycin, and 0.1  $\mu$ M bafilomycin). Peptide transport was quantified by fluorescence detection and transport activities were related to transport in the presence of MgATP (100%). Values are means of two to three independent experiments each with five replicates, error bars represent SD. Significance using the Student *t* test: (A) all *P*-values <0.0002; (B) *P*-values GTP, 0.0052; AMP-PNP, 0.0017; vanadate, 0.0002; (C) GTP, 0.0049; AMP-PNP, <0.0001; vanadate, 0.028; valinomycin, 0.0017.

inhibitor of the vacuolar H<sup>+</sup>-ATPase, did not inhibit the transport of the 9, 18, and 27 aa peptides. Interestingly, valinomycin even had a promoting effect on the uptake of the 27 aa peptide. Vanadate, an efficient inhibitor for most ABC-type protein-mediated transport processes, also inhibited the ATP-driven peptide transport. This inhibition was more pronounced (80%) for the shorter peptides, while transport of the 27 aa peptide was only inhibited by 50% (Fig. 3).

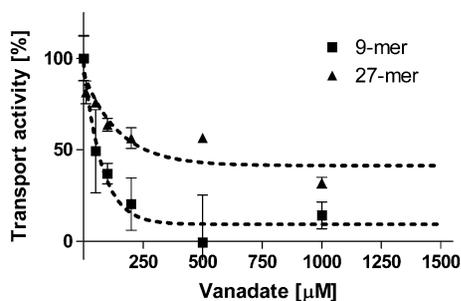
Since the inhibitory effect of vanadate at a concentration of 0.5 mM was different for short and long peptides, we wondered whether the concentration-dependence of the inhibition by vanadate was different for the 9 aa and 27 aa peptides. Raising the vanadate concentration did not consistently lead to a stronger inhibition than observed at 0.5 mM (Fig. 4). Furthermore, the concentration required to inhibit the transport rate to 50% of the maximal vanadate inhibition (IC<sub>50</sub>), were 75 μM and 80 μM, for the 9 aa and 27 aa peptides, respectively. Similar IC<sub>50</sub> values have already been reported for other ABC-mediated transport processes (Wolters *et al.*, 2005).

The affinity for MgATP has also been determined using the 9 aa peptide. The data fitted to the Michaelis–Menten equation revealed an apparent  $K_m^{\text{MgATP}}$  value of  $146.6 \pm 93.7$  μM (Fig. 5), a value which is similar to the affinity reported for other ABC transporters (Rea *et al.*, 1998).

#### Concentration-dependency of the peptide transporter and impact of the peptide length

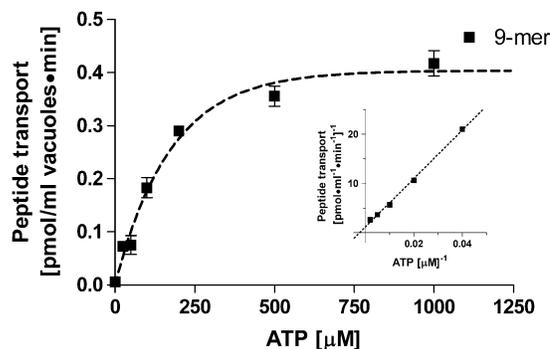
As shown in Fig. 6, transport rates for the 9 aa peptide followed Michaelis–Menten kinetics. As this experiment required a large amount of peptide, increasing the concentration until reaching saturation was not possible. From the data available, an apparent  $K_m^{\text{pep}}$  of about 15 μM and a maximal transport activity ( $V_{\text{max}}$ ) of about 8 pmol μl<sup>-1</sup> min<sup>-1</sup> were determined.

Furthermore, it was of interest to investigate how the transport of the 9 aa peptide was affected by peptides of different length. To this end, uptake experiments were

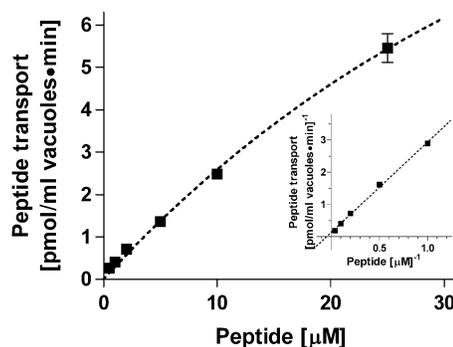


**Fig. 4.** Inhibition of 9 aa and 27 aa peptide uptake rates as a function of the orthovanadate concentration. Uptake of fluorescein-labelled 9 aa (black squares) and 27 aa (black triangles) peptides into barley mesophyll vacuoles was determined in the presence of increasing orthovanadate concentrations. Transport assays were carried out in the presence of 4 mM MgATP. Uptake in the absence of orthovanadate was set as 100%.

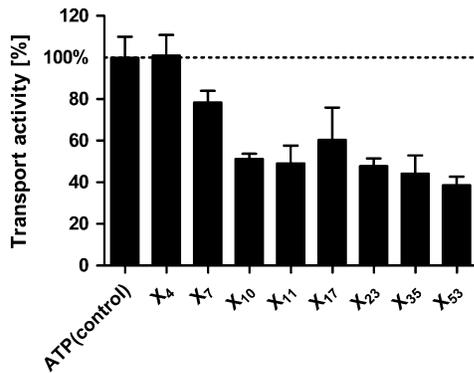
performed using 2 μM of the fluorescent 9 aa peptide in the presence of 20 μM of unlabelled randomized peptide libraries of increasing size ( $X_i$ ,  $i=4, 7, 10, 11, 17, 23, 35$ , and 53 residues; Fig. 7; Uebele *et al.*, 1997). The advantage of randomized peptide libraries is that they do not rely on a single sequence and hence reflect the competition by peptides unrelated to the fluorescent peptide used in uptake studies. The transport activity of the labelled peptide was not reduced in the presence of small peptides of four amino



**Fig. 5.** MgATP concentration dependent uptake of the 9 aa peptide. Uptake of the 9 aa peptide is dependent on the MgATP concentration. ATP dependency of the fluorescein-labelled 9 aa peptide (black squares) transport activity was established by increasing MgATP concentrations. Barley mesophyll vacuoles were incubated for 20 min in the presence of 2 μM 9 aa peptide. MgATP concentrations were kept constant by the addition of phosphocreatine (10 mM) and phosphocreatine kinase (16 U ml<sup>-1</sup>). Peptide transport was quantified by fluorescence detection and inhibition of the transport activity was related to transport in the absence of vanadate (100%). Results are means of two experiments per peptide. Each concentration is the mean of two independent experiments, each with five replicates and error bars represent SD.



**Fig. 6.** Concentration-dependent uptake of the 9 aa peptide in barley mesophyll vacuoles. Vacuoles were incubated for 20 min in the presence of 4 mM MgATP and fluorescein-labelled peptide (RRYC(φ)KSTEL). Concentration dependence of the peptide transport activity was determined by increasing the peptide concentration. The data were fitted to the Michaelis–Menten equation. Peptide transport was quantified by fluorescence detection. Each concentration is the mean of five replicates and error bars represent SD.



**Fig. 7.** Inhibition of the uptake of the 9 aa peptide by peptides of increasing size. Transport activities were determined by incubating barley mesophyll vacuoles in the presence of the fluorescein-labelled 9 aa peptide (RRYC( $\phi$ )KSTEL), 4 mM MgATP, and one of the non-labelled randomized peptide libraries of different length ( $X_i$ ,  $i=4,7,10, 11, 17, 23, 35$ , and 53 residues, see the Materials and methods). The concentration of labelled peptide was 2  $\mu$ M, non-labelled peptides were added at a 10-fold excess (20  $\mu$ M) and incubation time was 20 min. Peptide transport was quantified by fluorescence detection and transport rates were related to transport in the absence of a non-labelled peptide (100%). The data represent means of two to three independent experiments each with five replicates for each  $X_i$  aa peptide, error bars represent SD.

acids. The peptide with 7 aa exhibited 20% inhibition, while the peptide with 10 and more aa inhibited uptake of the 9 aa peptide by about 50%. This value can be expected taking into account a  $K_m$  of 15–20  $\mu$ M for the 9 aa peptide. These experiments indicate that the transporter does not strongly discriminate between different peptides with the same size. Longer peptides had the tendency to be slightly more inhibitory (Fig. 7). This inhibition study suggests that while the transport activity increases with the size of the peptide (Fig. 7) the affinity of the transporter for the longer chain peptides remains the same.

## Discussion

Peptides play an important role not only in nitrogen nutrition but also in various processes occurring within living cells (Higgins and Payne, 1981; Stacey *et al.*, 2002b). Despite the identification of a large number of putative peptide transporters in the *Arabidopsis* genome, little is known about their activity, localization, and involvement in whole plant metabolism. All plant peptide transporters described so far transport small peptides up to five amino acids. Only AtOPT6 was shown to transport peptides as long as 13 amino acids (Pike *et al.*, 2009). Large peptides may be generated by endoproteases as well by the proteasome complex. The universal ubiquitin–proteasome complex pathway is the best-defined protein degradation pathway (Smalle and Viestra, 2004). The proteasome complex exhibits three proteolytic activities, a chymotrypsin-like, a trypsin-like, and a peptidyl-glutamyl peptide-

hydrolysing activity. Combined together, these proteases generate peptides of a size between 3 and 30 amino acids. The ubiquitin–proteasome complex pathway together with the less-well characterized cytoplasmic and plastid degradation pathways produce the major part of the peptides found in the plant cell (Sakamoto, 2006). So far, the subsequent step of this protein turnover process, i.e. the final degradation of the resulting peptides leading to the production of amino acids, which can be reused for *de novo* protein synthesis, is less understood. Since a long time, the presence of hydrolytic enzymes in vacuoles had been demonstrated (Boller and Kende, 1979). It was, however, not clear whether peptides have to be imported into the vacuole for their final degradation.

In contrast to plants, transporters for long-chain peptides have been well characterized in animals. These transporters belong to the so-called TAP (Transporter associated with Antigenic Processing) family. In humans, TAP1 (ABCB2) and TAP2 (ABCB3) form a heterodimer which transports peptides generated by the proteasome complex into the ER where they are loaded onto a newly synthesized MHC class I complex. The MHC I-peptide complex is exported to the cell surface to be exposed to T-cells. By means of its function, this heterodimer was shown to play a central role in the immune system. (Trowsdale *et al.*, 1990; Bahram *et al.*, 1991, Higgins, 1992; Abele and Tampé, 2004). In mammals, the third member of this family is the so-called TAP-like protein, which forms homodimers and was shown to be a peptide transporter located in the lysosomal membrane (Wolters *et al.*, 2005; Demirel *et al.*, 2007).

The finding of TAP-like transporters in the plant vacuolar membrane by different proteomic approaches encouraged the investigation of the vacuolar transport activity for larger peptides (Endler *et al.*, 2006). Peptides were chosen with similar amino acid sequences as used to characterize the human TAP heterodimer mediating peptide transport (Lankat-Buttgereit and Tampé, 2003). All three fluorescein-labelled peptides tested were transported across the tonoplast in a strictly ATP-dependent way. As expected for an ABC-transporter-mediated uptake, GTP but not AMP-PMP could substitute for ATP. Furthermore, peptide transport was inhibited by vanadate, but not by bafilomycin or valinomycin. Vanadate inhibition was more pronounced for shorter peptides, whereas the transport of the 27 aa peptide was inhibited only by about 50%. Valinomycin increased the transport activities in the case of larger peptides (Fig. 3). This stimulating effect of valinomycin could be related to the excess of positive charges, as the longer peptides used in this study contained an increased number of positive charges. Destroying the trans-vacuolar membrane potential may, therefore, reduce the energy for the transfer of the peptides and this would be most pronounced for the 27 aa peptide, since it contains six net positive charges.

Our data suggest that the vacuole has at least one ABC-type protein that transports peptides from the cytosol into the vacuole. The transporter exhibited an apparent affinity

of approximately 15  $\mu\text{M}$  for 9 aa peptides, which is similar to that of the human ABCB9/TAPL (6.8  $\mu\text{M}$ ) (Wolters *et al.*, 2005). This high affinity of the transporter indicates an efficient transport of the peptides from the cytosol into the vacuole. It is likely that the peptides transported into the vacuole are subsequently degraded to free amino acids by vacuolar endoproteases and exopeptidases. It is therefore hypothesized that the proteasome and perhaps other, so far unknown, cytosolic proteolytic activities produce peptides which are readily transported into the vacuole in order to be degraded and recycled to sustain plant metabolism. Whether peptides generated by plastidic proteases are exported into the cytoplasm and finally transported into the vacuole is so far unknown. TAP-like plant transporters have been localized in the vacuolar membrane using a GFP fusion protein as well as by proteomic approaches (Yamaguchi *et al.*, 2002; Endler *et al.*, 2006). Further studies will show whether these TAP-like proteins act as long-chain peptide transporters in plants as well. *Arabidopsis* AtTAP2/AtABCB27/ASL1 and the barley TAP, HvID17, have been proposed to be related with other processes. HvID17 expression is strongly induced by iron deficiency and therefore HvD17 has been proposed to be implicated in iron homeostasis (Yamaguchi *et al.*, 2002). However, this hypothesis is based only on the strongly increased expression of HvID17 during iron starvation and it cannot be excluded that iron starvation leads to an increased protein turnover and peptide import into the vacuole. AtTAP2/AtABCB27 has been proposed to be involved in aluminium tolerance, since the corresponding knock-out mutant exhibited reduced root growth when grown on  $\text{Al}^{3+}$  containing medium (Jaquinod *et al.*, 2007; Larsen *et al.*, 2007). Further studies are required to identify which transporter is responsible for the long-chain peptide transport and which role these transporters play in nitrogen metabolism of the plant and Dr. Ulrike Schmidt for helpful discussions.

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